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Gene flow in the European corn borer *Ostrinia nubilalis*: implications for the sustainability of transgenic insecticidal maize

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Strategies proposed for delaying resistance to *Bacillus thuringiens*is toxins expressed by transgenic maize require intense gene flow between individuals that grew on transgenic and on normal (referred to as refuges) plants. To investigate gene flow in the European corn borer, *Ostrinia nubilalis* (Hübner), the genetic variability at 29 sampled sites from France was studied by comparing allozyme frequencies at six polymorphic loci. Almost no deviations from Hardy–Weinberg expectations occurred, and a high stability of allelic distribution was found among samples collected in the same site over two or three different generations, indicating a high stability of the genetic structure over time. The overall genetic differentiation was low at the region and whole country level, suggesting a high and homogeneous gene flow. These results are discussed in relation to the sustainability of transgenic insecticidal maize.

Keywords: *Ostrinia nubilalis*; European corn borer; pest management; genetically modified organisms; population genetics

1. INTRODUCTION

Sustainability of transgenic cultivars requires a solid understanding of the target insect's populations (Gould 1998). However, we presently lack the necessary information to use transgenic insecticidal cultivars in ways that would avoid rapid genetic adaptation by target pests (Roush 1997; Gould 1998; McGaughey *et al*. 1998). Comins (1977) first showed that random gene exchange between selected and unselected (refuge) insect populations in a patchwork can delay the evolution of resistance. This approach, coupled with high-dose applications, appeared to be one of the best strategies for resistance management for many transgenic crops producing *Bacillus thuringiensis* toxins (*Bt* crops) (Alstad & Andow 1995; Gould 1998). To be effective the high-dose/refuge strategy was shown to require three main components (Andow & Alstad 1998). First, resistance must be functionally recessive so that heterozygous individuals for a resistance allele are killed by the toxin expressed by the plant tissues. Second, resistance alleles must be rare ($p<10^{-3}$) so that only few ($p^2<10^{-6}$) homozygotes would survive on transgenic cultivars. Third, the high-dose/refuge strategy implies that resistant insects selected in *Bt* crops must mate randomly or preferentially with susceptible insects preserved on non-*Bt* crops (Alstad & Andow 1995).

Bt crops such as cotton and maize transgenic varieties are toxic to many Lepidoptera, including Noctuidae and Pyralidae. *Ostrinia nubilalis* Hübner (Pyralidae), the European corn borer (ECB), is one of the most injurious pests of maize in North America and Europe. A recent study has shown that the first component of the high-dose/refuge strategy may not be fulfilled as some mutant alleles of *O. nubilalis* conferred a dominant resistance to *Bt* toxins (Huang *et al*. 1999). The second component of the strategy—i.e. the frequency of resistance alleles prior to the introduction of transgenic maize—has not been evaluated yet (but see Andow & Alstad 1998; Andow *et al*. 1998). The purpose of the present study was to investigate the third component of the high-dose/refuge strategy, which implies intense gene flow between ECB populations. Allozyme polymorphism is well suited for population studies and has been used to investigate the genetic population structure in several migrant Noctuidae species (e.g. Nibouche *et al*. 1998). Despite a few investigations of ECB allozyme variability (Harrison & Vawter 1977; Cardé *et al*. 1978; Glover *et al*. 1991; Wang *et al*. 1995), an analysis of population structure has never been performed on this pest species. This is of primary importance in order to provide precise scientific data to the debates on the sustainability of transgenic maize. Therefore the present study was devoted to assess the extent of gene flows in *O. nubilalis* within and between 29 sites localized all over France.

2. MATERIAL AND METHODS

(a) *Sampling localities*

Twenty-nine different sites were sampled in France (table 1 and figure 1). Samples were collected as larvae diapausing into maize stalks. For samples collected from Landes and Ile-de-France regions, larvae were kept in the stalk during the winter to allow diapause to be completed. Larvae were removed from the stalk during the spring and reared on an artificial diet until pupation. After emergence, adults were sexed and frozen at $-80\,^{\circ}$C. For the other samples, larvae were scored for their sex and directly frozen at $-80\,^{\circ}$C.

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Table 1. *Characteristics of samples of* O. nubilalis*: location, date of sampling and number* (N) *of ECBs analysed*

geographical region	region	department	locality	population	latitude	longitude	date (month/year)	N
south	Aquitaine	Landes	Heugas	Heu	43°38′ N	1°04′ W	10/1998	40
			Arthez-d'Armagnac	Art	43°53′ N	0°15′ W	10/1995	33
							10/1996	8
							09/1997	48
			Eyres-Montcubes	Eyr	43°43′ N	0°33′ W	10/1996	17
							10/1997	19
			Audon	Aud	43°48′ N	0°49′ W	10/1996	50
							10/1997	47
			Souprosse	Dub	43°46′ N	0°42′ W	10/1995	18
							10/1996	34
							10/1997	12
				Dup	43°47′ N	0°42′ W	10/1996	24
							10/1997	55
				Laf	43°46′ N	0°42′ W	10/1996	46
							09/1997	51
				Lap	43°46′ N	0°43′ W	10/1995	28
	Midi-Pyrénées	Haute-Garonne	Baziège	Baz	43°27′ N	1°37′ E	10/1997	39
	Languedoc-Roussillon	Aude	Carcassonne	Car	43°29′ N	2°03′ E	10/1998	40
	Catalonia (Spain)	—	Lleida	Esp	41°37′ N	0°36′ E	11/1998	40
north-west	Pays de la Loire	Maine-et-Loire	Le Loroux Beconnais	Lor	47°31′ N	0°53′ E	10/1998	40
	Poitou-Charentes	Deux-Sèvres	Lezay	Lez	46°16′ N	0°00′ E	10/1998	40
	Ile-de-France	Seine-et-Marne	La Brosse Montceaux	Run	48°23′ N	2°52′ E	10/1998	40
		Seine-et-Marne	St Germain/Ecole	Ger	48°31′ N	2°29′ E	10/1996	49
	Haute-Normandie	Eure	Mesnil-Andé	Mes	49°16′ N	1°13′ E	10/1998	40
	Picardie	Somme	Le Bosquel	Pic	49°44′ N	2°14′ E	10/1998	40
north-east	Bourgogne	Saône-et-Loire	Craman	Roi	47°01′ N	5°46′ E	11/1998	40
			Fontaines	Fon	46°51′ N	4°46′ E	11/1998	40
	Franche-Comté	Doubs	Roulans	Rou	47°19′ N	6°14′ E	11/1998	40
		Jura	Parcey	Par	47°02′ N	5°30′ E	11/1998	40
		Territoire de Belfort	Croix	Cro	47°26′ N	6°57′ E	11/1998	40
		Haute-Saône	Cussey l'Ognon	Cus	47°20′ N	5°56′ E	11/1998	40
			Chambornay les Pins	Cha	47°18′ N	5°56′ E	11/1998	40
				Ogn	47°18′ N	5°56′ E	11/1998	40
			Sauvagney	Sau	47°18′ N	5°56′ E	11/1998	40
			Valay	Val	47°20′ N	5°38′ E	11/1998	40
	Nord-Pas-de Calais	Nord	Solesmes	Sol	50°11′ N	3°28′ E	10/1998	40
	Alsace	Bas-Rhin	Wolschheim	Wol	48°42′ N	7°26′ E	10/1998	40

(b) *Electrophoresis*

Each moth or larva was homogenized in 150 µl Tris–EDTA buffer (pH 6.8) after discarding the head. Horizontal starch gel electrophoreses of homogenates were carried out using Tris–borate–EDTA (pH 8.6) buffer systems (Pasteur *et al*. 1987). Enzymes were revealed by adapting staining recipes described by Pasteur *et al*. (1987) except for the triose phosphate isomerase (TPI) protocol that was adapted from Glover *et al*. (1990). Twenty-eight enzyme systems were studied but only six were kept for their non-equivocal genetic interpretations, their absence of variation in patterns between adults and larvae and their polymorphisms. These enzyme systems were the phosphoglucomutase (PGM, EC 2.7.5.1), the mannose 6-phosphate isomerase (MPI, EC 5.3.1.8), the hydroxybutyrate dehydrogenase (HBDH, EC 1.1.1.30), the glucose-phosphate isomerase (GPI, EC no. 5.3.1.9), the aspartate-amino-transferases (AAT, EC 2.6.1.1) and the TPI (EC 5.3.1.1).

(c) *Data analysis*

For each sampled site, the allelic frequencies (available upon request to the first author), the mean number of alleles (N_{all}), the observed heterozygosity (H_o), the gene diversity (H_e) and the $\hat{f}$-values (i.e. the F_{is} estimates according to Weir & Cockerham 1984) were estimated using the software Fstat 2.3 (Institute of Ecology, Lausanne, Switzerland) (J. Goudet). N_{all}, H_o, H_e and $\hat{f}$ were calculated over all individuals and loci except for *Tpi*. Indeed *Tpi* is located on the sexual Z chromosome (Glover *et al*. 1990) and females are heterogametic (ZW) whereas males are homogametic (ZZ). Thus at this locus females are hemiploid whereas males are diploid. Tests for deviations from Hardy–Weinberg

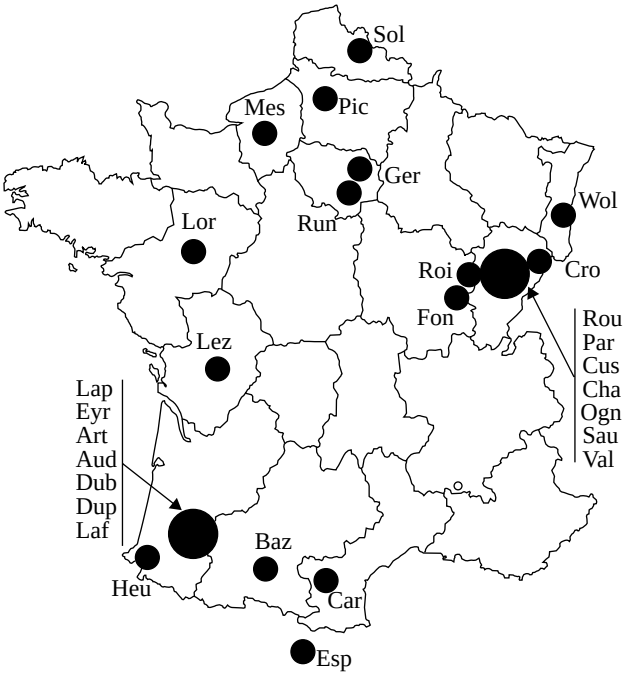

Figure 1. Geographical location of the 29 sampled sites reported in table 1 (for definitions, see table 1).

expectations at each locus and for genotypic linkage disequilibria among loci were computed within each sampled site with the software GENEPOP 3.1d (Raymond & Rousset 1995).

For six sampled sites, two or three samples were collected over three years (see table 1). The temporal variation of allelic frequencies within each site was analysed between each pair of samples and over all samples. Homogeneity tests were computed as exact tests (GENEPOP 3.1d) for each locus and a Fisher's method for combining independent results (each locus) was used to test the null hypothesis over all the loci.

The genetic structure among sites, for each of the six polymorphic loci and over all the loci, was analysed by testing for genotypic differentiation by using exact tests and computing the estimator $\hat{\theta}$ of F_{ST} according to Weir & Cockerham (1984) using GENEPOP 3.1d (Raymond & Rousset 1995). Isolation by distance patterns (Slatkin 1993) was also tested by analysing the independence between geographical and genetic distances at various geographical levels. The null hypothesis of independence between geographical and genetic distances was tested against the hypothesis of positive correlation expected under isolation by distance, estimated as Spearman's rank correlation coefficient. The observed correlation coefficient was compared with the distribution of correlation coefficients obtained from Mantel-like permutations of the genetic $(\hat{\theta}/(1-\hat{\theta}))$ and geographical (ln(geographical distance)) matrices following Roussett (1997) as included in GENEPOP 3.1d. All the among-sites analyses were conducted at different geographical levels as the sampled sites may be clustered into three distinct geographical areas (see table 1): the south of France (11 sites: Heu, Art, Eyr, Aud, Dub, Dup, Laf, Lap, Baz, Car, Esp), and the north of France subdivided into two regions—north-east (11 sites: Roi, Fon, Rou, Par, Cro, Cus, Cha, Ogn, Sau, Val, Wol) and north-west (seven sites: Lor, Run, Ger, Lez, Mes, Sol, Pic). In addition, hierarchical analyses of population structure were performed by partitioning $\hat{\theta}$ into $\hat{\theta}_S$ and $\hat{\theta}_P$ indicating the genetic differentiation of sampled sites within region and among regions, respectively (Weir & Cockerham 1984), by using the software TFPGA (Miller 1997).

3. RESULTS

Over the 29 sampled sites analysed, four, seven, four, five, eight and five alleles were observed at loci *Tpi*, *Gpi*, *Hbdh*, *Aat*, *Mpi* and *Pgm*, respectively. This allowed population genetics analyses to be conducted at the geographical scale under study.

Exact tests for genotypic linkage disequilibria resulted in one significant value ($p=0.03$, *Gpi* and *Aat*) out of ten combined tests for each locus pair across all sampled sites. At the within-site level (table 2), the mean number of alleles ranged from 2.20 to 4.00 over the five autosomal loci used. All of the sampled sites were polymorphic at one locus at least. The observed and expected heterozygosities were almost identical and ranged from 0.19 to 0.33 and 0.23 to 0.32, respectively, across sites. The estimates of $\hat{f}$-values did not reveal a large excess or deficit in heterozygotes, and deviations from Hardy–Weinberg expectations across the five loci were significant in only two out of 29 sampled sites (i.e. in Heu ($p=0.03$) and Sol ($p=0.05$)); however, these did not remain significant ($p>0.05$) when taking into account multiple tests (Bonferroni test, Holm 1979).

There was no evidence for temporal variation between each pair of samples and over all samples at each of the sites that were sampled on more than one occasion (namely, Art, Aud, Dub, Dup, Eyr and Laf, see table 1). The probability values associated with the global test (Fisher's method) over all loci and sampling dates were always higher than 0.342. Classical homogeneity tests, as exact tests, do not account for genetic drift and sampling drift effects and alternative procedures have to be used (Waples 1989; Viard *et al.* 1997). Here, we failed to detect any significant variation of allelic frequencies with all the analyses conducted. The same results held when analysing the loci separately or with any combination of sampling dates. This result demonstrated a high stability of the allelic distribution over time (two to three years, that is four to six generations).

The overall differentiation among sites although significant ($p<10^{-5}$) is characterized by a very low mean $\hat{\theta}$-value (0.011) (table 3). Such low $\hat{\theta}$-values were also observed at a regional scale (table 3) with the lowest value observed in the south of France ($\hat{\theta}=0.003$).

The Mantel-like tests for independence between geographical and genetic distances were significant over the whole data set ($p=0.004$). When analysing the data set at a regional scale, only one significant probability value was observed, namely the north-west of France.

The hierarchical analysis of the distribution of the genetic variability demonstrated that the larger part of the genetic differentiation occurred at the within-site level. When comparing north versus south sites, $\hat{\theta}_S$ and $\hat{\theta}_P$ equalled 0.012 (significantly different from zero) and 0.002, respectively. When comparing north-west, north-east and south, $\hat{\theta}_S$ and $\hat{\theta}_P$ equalled 0.012 and 0.003, respectively, and both were significantly different from zero.

4. DISCUSSION

Our study of the genetic structure of *O. nubilalis* populations in France, based on allozyme markers, reveals a high degree of gene flow occurring within and between

Table 2. *Within-site genetic parameters*

(Each sampled site is characterized by its name. The mean number of alleles (N_{all}), the observed heterozygosity (H_o) and the gene diversity (H_e) are given as well as the estimates of the $\hat{f}$-values and the p-value of the test for deviation from Hardy–Weinberg expectation per population over five loci (*Gpi*, *Hbdh*, *Aat*, *Mpi*, *Pgm*), see text for details.)

	N_{all}		H_o		H_e			
	mean	s.e.	mean	s.e.	mean	s.e.	$\hat{f}$	p
south								
Heu	2.40	1.34	0.21	0.24	0.25	0.26	0.19	0.03
Art	3.80	0.84	0.25	0.22	0.27	0.26	0.09	0.40
Eyr	2.80	1.10	0.23	0.20	0.24	0.22	0.05	0.92
Aud	3.40	1.67	0.28	0.28	0.27	0.25	−0.05	0.31
Dub	4.00	1.41	0.31	0.30	0.28	0.26	−0.09	0.41
Dup	3.20	1.79	0.22	0.22	0.24	0.25	0.07	0.88
Laf	3.60	1.52	0.23	0.22	0.27	0.26	0.13	0.14
Baz	2.40	0.89	0.23	0.20	0.26	0.26	0.15	0.51
Car	2.60	1.14	0.25	0.25	0.24	0.24	−0.06	0.90
Esp	2.80	0.45	0.27	0.23	0.25	0.21	−0.07	0.48
north-west								
Lor	2.60	1.14	0.28	0.23	0.30	0.26	0.07	0.45
Run	2.80	1.30	0.33	0.21	0.33	0.24	0.03	0.78
Ger	3.00	1.22	0.21	0.20	0.25	0.26	0.16	0.22
Lez	2.80	1.30	0.31	0.25	0.30	0.22	−0.02	0.36
Mez	3.00	1.41	0.32	0.29	0.29	0.25	−0.09	0.92
Pic	3.00	1.22	0.32	0.27	0.31	0.26	−0.03	0.36
north-east								
Sol	2.60	1.14	0.28	0.26	0.29	0.27	0.07	0.05
Roi	2.80	1.48	0.33	0.23	0.32	0.23	−0.03	0.91
Fon	2.60	1.14	0.25	0.19	0.30	0.24	0.16	0.34
Rou	3.20	0.84	0.26	0.24	0.27	0.25	0.02	0.97
Par	3.40	0.89	0.29	0.23	0.30	0.24	0.03	0.81
Cro	2.80	0.45	0.24	0.17	0.23	0.17	−0.02	0.99
Cus	3.00	0.71	0.19	0.13	0.23	0.16	0.19	0.38
Cha	3.00	0.71	0.29	0.23	0.29	0.24	0.03	0.47
Ogn	2.20	0.84	0.23	0.20	0.27	0.24	0.14	0.57
Sau	2.80	1.79	0.28	0.29	0.27	0.27	−0.03	0.46
Val	2.80	1.10	0.24	0.21	0.30	0.28	0.19	0.40
Wol	3.20	0.45	0.32	0.22	0.32	0.21	0.00	0.30

sampled sites. At the site level no significant departure from Hardy–Weinberg expectations was noted when considering multiple statistical tests. This result associated with the high stability of allelic distribution over generations in six sites from the south of France, strongly suggests a high stability of the observed genetic structure and panmixia among individuals collected in each sampled locality. Moreover, independently of the geographical scale considered (regions or country) very little genetic differentiation was observed between sites. Thus, it can be concluded that there is extensive gene flow within and among populations of *O. nubilalis* over the whole territory of France. The only pattern of isolation by distance occurred in the north-west area. This may be due to the fact that maize fields are fewer and more dispersed in the north-west region compared with the south and the north-west areas.

The present results can be compared with those obtained with Noctuids moth pests. As observed in our study, the genetic differentiation between samples of *Helicoverpa armigera* within a region (less than 50 km in diameter) was low (Nibouche *et al*. 1998). For instance, in south-east France, there was no significant difference in allele frequencies, and samples appeared to belong to the same panmictic population. In contrast Korman *et al.* (1993), studying the variability of *Heliothis virescens* from the southern USA, concluded that panmictic populations spread over areas of less than 8 km in diameter.

In North America *O. nubilalis* displays several races morphologically indistinguishable which have distinct voltinism, host-plant range and sex pheromone communication systems (Roelofs *et al*. 1985; Hudon *et al*. 1989). Only a few studies have been devoted to understanding the genetic relationships between these races. Harrison & Vawter (1977) and Cardé *et al.* (1978) found that two sympatric pheromonal races displayed slight differences in their allelic frequencies. Using the *Tpi* locus, Glover *et al.* (1991) revealed that gene flow was asymmetrical between ECB pheromonal races. Races with distinct pheromones and voltinism have also been reported in France (Anglade *et al*. 1984; Stengel & Schubert 1982). In the present study all the samples were collected on maize stalk, avoiding a mixture of races potentially specialized on various plant hosts. However, the pheromone communication systems and the voltinism were not investigated. In France the voltinism of the ECB has both a genetic

Table 3. *Genetic differentiation*

($\hat{\theta}$-values estimated according to Weir & Cockerham (1984) among sites within and among regions. IBD corresponds to isolation by distance.)

	Tpi	*Gpi*	*Hbdh*	*Aat*	*Mpi*	*Pgm*	all loci	*p*-value	IBD (*p*-value)
France	0.004	0.004	0.016	0.005	0.020	0.041	0.011	$<10^{-5}$	0.004
south	0.003	0.002	0.000	0.000	0.004	0.009	0.003	0.031	0.243
north	0.003	0.002	0.028	0.011	0.034	0.032	0.015	$<10^{-5}$	0.142
north-east	0.007	0.000	0.021	0.005	0.041	0.013	0.015	$<10^{-5}$	0.291
north-west	0.000	0.000	—[a]	0.022	0.023	0.033	0.012	$<10^{-5}$	0.014

[a] Irrelevant (monomorphic locus).

and an environmental component (Stengel & Schubert 1982). In general, populations from southern France are bivoltine whereas those from northern France are monovoltine. We therefore could have expected a differentiation between the northern and southern parts of France, which is not the case from the present data set. The most differentiated areas are north-west compared with north-east.

Gene flow within and between ECB populations is a key component for the sustainability of transgenic insecticidal maize. The intensive gene flow revealed by the present study has a double effect. It should result (i) in the spreading of resistance alleles over a large geographical area, and (ii) in a reduction in local resistance to *Bt* toxins because of the presence of susceptible immigrants from the non-*Bt* maize. This latter component is one of the three main assumptions underlying the high-dose/refuge strategy for delaying the evolution of resistance to *Bt* toxins (Alstad & Andow 1995; Andow *et al*. 1998; Gould 1998). Indeed this strategy assumes random mating between moths grown on transgenic and normal (i.e. refuge) cultivars. To avoid the movement of individual larvae from *Bt* maize to normal maize (or weedy hosts) within the field that would compromise the high-dose strategy (Gould 1998), implementation of the refuge in separate areas has been recommended. However, it was also noted that, if the spatial distribution of *Bt* crops and refuges is very large relative to adult movements of a target pest species (e.g. farm to farm), most resistant individuals surviving *Bt* toxic plants might not move far enough to mate with susceptible moths and vice versa. Our results suggest that this risk is low due to the high observed gene flow (and therefore migration), and thus that local adaptation to the toxin is not likely to occur rapidly, if no other biological traits than migration are relevant.

That random mating and high gene flow occur within and among actual populations does not ensure that random mating will also exist between resistant and susceptible moths. Gould (1998) pointed out that resistant individuals that feed on high-dose *Bt* maize may develop more slowly than susceptible moths. This temporal asynchrony may lead susceptible ECBs to complete mating before resistant moths emerged. Such an asynchrony has been just reported for *Bt*-resistant individuals of the pink bollworm moths *Pectinophora gossypiella* (Liu *et al*. 1999). Moreover even if resistant ECBs were receptive to mating with susceptible individuals, they might be less attractive than susceptible ECBs as previously shown for the diamondback moth *Plutella xylostella* (Groeters *et al*. 1993).

Finally for determining how the refuge should be organized we need more data on how far adults move before they mate. *O. nubilalis* has been observed moving to the periphery of maize fields before mating (Showers *et al*. 1976; Stockel *et al*. 1985) and can move over large distances in a single generation (Chiang 1972) although this may not be always the case (Derrick *et al*. 1992). More studies are required at the field level before the optimal spatial scale for a mixture of *Bt* and non-*Bt* maize can be determined.

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